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Re: IMMORTALIZED HEPATOCYTES
Our Docket No.: 8028-005-PR

Dear Sir or Madam:

Enclosed please find the following documents related to the above-identified matter:

1. Provisional Application for Patent Cover Sheet (PTO/SB/16);
2. Fee Transmittal (PTO/SB/17);
3. Specification/claims;
4. Figures;
5. Self-addressed, stamped postcard.

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Sincerely yours,

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Enclosures

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.	EV 117250495 US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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☑ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Immortalized Hepatocytes

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ENCLOSED APPLICATION PARTS ***(check all that apply)***

☑ Specification *Number of Pages*	41	☐ CD(s), Number	
☑ Drawing(s) *Number of Sheets*	4	☐ Other (specify)	
☐ Application Data Sheet. See 37 CFR 1.76			

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Respectfully submitted,

SIGNATURE Jessica S. Mitchell

Date 10/10/2003

TYPED or PRINTED NAME Jessica S. Mitchell, Esq.

REGISTRATION NO. *(if appropriate)* 54,317

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Date: October 10, 2003 Express Mail Label No.: EV 117250495 US

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Attorney's Docket No.: 8028-005-PR

IMMORTALIZED HEPATOCYTES

GOVERNMENT GRANTS

This invention was made in part with United States government support under grant number 70-NANB7H3070 awarded by Advanced Technology Program of the United States Department of Commerce. The U.S. government has certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to novel virally-immortalized normal human hepatocyte cell lines.

BACKGROUND OF THE INVENTION

The safe and efficient production of novel therapeutic proteins represents an expanding market of the biopharmaceutical industry that is fueled by the recent completion of the Human Genome Project and by rapid technological advances in the

field of proteomics. Paulaus, A., *The reengineering of drug development in the genomics and proteomics era.* Am Clinical lab, 2001. **5**: p. 55-57.

Although many of these therapeutic proteins are mass-produced by recombinant technology, there are occasions where the commercialization of complex heterologous proteins, due to their being the products of multiple genes, are better accomplished by isolating the native form of the protein.

In addition, the production of therapeutic plasma proteins (TPP) by cell-based systems would avoid the hazards of blood-derived products, the most notable of which is viral contamination. Although, when processed correctly, blood-derived products are virtually free of transmitting viral infections, a perceived risk exists for the manufacturer, user, and patient. Indeed, the recent discovery of new strains of human immunodeficiency virus and the agents responsible for the transmissible spongioform encephalopathies, such as mad cow disease, exemplify the everlasting concern for blood-derived products. Collinge, J., et al., *Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD.* Nature, 1996. **383**(6602): p. 685-90.

Limitations of Recombinant Proteins as Therapeutic Drugs.

Currently, the majority of all proteins that have been approved for clinical and therapeutic use, with the exception of monoclonal antibodies, are mass-produced by recombinant protein technology. Although these products have been proven safe and effective, not all behave identically to their native counterparts. For example, recombinant factors (rF) VIII and IX are more rapidly cleared following infusion than their plasma derived counterparts. Shapiro, A., E. Berntorp, and M. Morfini,

Incremental recovery assessment and effects of weight and age in previously untreated patients treated with recombinant factor IX. Blood, 2000. **96 (suppl 1)**: p. 265a.

Recent findings suggest that this is the result of incomplete or inappropriate post-translational modification. The rapid clearance of the β-domain deleted form of factor VIII, which is used in the United States, is due to differences in phospholipid binding. In contrast, differences in sulfation at tyrosine 155 and phosphorylation of serine 158 of Factor IX, result in more rapid clearance of the clotting factor. White, C.G.I., A. Beebe, and B. Nielsen, *Recombinant factor IX.* Thromb Haemost, 1997. **78**: p. 261-265.

Clinically, more rapid clearance of these clotting factors means potentially more frequent and higher dosages depending upon the patient population. Although one strategy to circumvent these shortcomings is to use plasma-derived proteins, there are also perceived risks, as mentioned above, associated with this approach.

Significant Unmet Need for Therapeutic Proteins

Hemophilia A (Factor VIII deficiency) and hemophilia B (Factor IX deficiency) are bleeding disorders that are inherited as x- linked recessive traits. Thus, both affect males almost exclusively. Both hemophilia A and hemophilia B are heterogeneous conditions with variable degrees of clinical expression. Hemophilia A is far more common, occurring in 1 in (5000 to10,000) males in the United States. Soucie, J.M., B. Evatt, and D. Jackson, *Hemophilia Occurrence in the United States.* American Journal of Hematology, 1998. **59**: p. 288-294.

In contrast, the incidence of hemophilia B is 0.25 in 10,000 males. Currently, plasma-derived and recombinant Factor VIII and IX concentrates are used for the lifetime treatment

of hemophilia. It is estimated that three-quarters of the worldwide hemophilia population receive little or no treatment due to a shortage of this TPP. Thus, there is a clear need for fully functional, fully native blood-clotting factors that overcome the shortcomings of recombinant or blood-derived TPPs.

α-1-antitrypsin (AAT) is a human blood protein whose prime physiological target is neutrophil elastase. Severe AAT deficiency (hereditary emphysema) is thought to affect around 150,000-200,000 individuals in Europe and US. Donohue, T.M., et al., *Synthesis and secretion of plasma proteins by the liver*, in *Hepatology: A Textbook of Liver Disease*, D. Zakim and T.D. Boyer, Editors. 1990, W.B. Sounders Company: Philadelphia. p. 124-137.

Many respiratory diseases including AAT congenital deficiency, cystic fibrosis and chronic obstructive pulmonary disease are characterized by an imbalance of AAT and elastase in the lung. An abundance of elastase is thought to contribute to damage of the pulmonary epithelium. Administration of supplemental AAT is therefore expected to alleviate the deleterious effects of elastase in the lung in these diseases.

Approximately one in 2000 children is born with the CF genetic defect in the Western Hemisphere. Currently, there is only one plasma-derived AAT licensed in the United States, which has been in very limited supply. Many of the diagnosed patients have therefore not had access to AAT treatment. Despite the large body of evidence of the clinical efficacy of AAT to treat general inflammatory conditions, its use has been restricted due to the limited availability of the product. Thus, there is a clear need for

fully functional, fully native AAT that overcome the shortcomings of recombinant or blood-derived TPPs.

Efficacy of IαIp as a Treatment of Sepsis.

Sepsis is a disease characterized by an overwhelming systemic response to infection, which can rapidly lead to organ dysfunction and ultimately death. Sepsis can strike anyone and can be triggered by events such as pneumonia, trauma, surgery and burns, or by conditions such as cancer or AIDS. Once triggered, an uncontrolled cascade of coagulation, impaired fibrinolysis (clot breakdown), and inflammation fuels the progression of sepsis. In the United States, sepsis is the leading cause of death in the noncardiac intensive care unit and the 11^{th} leading cause of death overall.

Each year, over 700,000 new cases of sepsis are diagnosed. Currently, treatment for sepsis is limited to attempts to manage the underlying infection and supportive therapy if the infection leads to organ dysfunction. Despite intensive medical care, up to 50% of patients still die from this illness. Rangel-Frausto, M.S., et al., *The natural history of the systemic inflammatory response syndrome (SIRS): a prospective study.* JAMA, 1995. **273**: p. 117-123.

Given the intensive and prolonged care necessary to treat patients with sepsis, the economic burden is profound. For decades, physicians treating patients with severe septic illness have searched for an effective addition to their available therapeutic arsenal (mainly antibiotics) that could reduce the high mortality rate associated with this disease. Many of the attempted therapeutic interventions in human sepsis have been based upon the premise that

circulating endotoxin is responsible for the critical clinical manifestations and morbidity of sepsis.

Indeed, some investigators have concluded that any adjunctive therapy is destined to fail because once the clinical signs of severe sepsis are present, irreversible organ injury has already occurred. Recently, a promising new class of therapeutic agents based on natural plasma proteins with anti-coagulative activities has recently appeared on the clinical horizon. In severe sepsis, the coagulation system is activated; an event evidenced by the presence of intravascular thrombi in vessels and tissue and the occurrence of disseminated intravascular coagulation. Large multicenter phase III studies of activated protein C (APC) and antithrombin III (AT-III) in sepsis were completed in early 2001.

Inter-α-inhibitor proteins (IαIp), natural serine protease inhibitors found in relatively high concentration in plasma have been shown to play roles in inflammation, wound healing and cancer metastasis reviewed by Bost et al. Bost, F., M. Diarra-Mehrpour, and J.P. Martin, *Inter-alpha-trypsin inhibitor proteoglycan family-a group of proteins binding and stabilizing the extracellular matrix.* Eur J Biochem, 1998. **252**: p. 339-346. The major forms of IαIp are inter-α-inhibitor (IαI, containing one light chain peptide called bikunin and two heavy chains) and pre-a-inhibitor (PαI, containing one light and one heavy chain).

Recently, a monoclonal antibody that recognizes the light chain of human IαIp (MAb 69.31) was developed by scientists and collaborators at Prothera Biologics (Providence, Rhode Island). Using MAb 69.31 in a competitive ELISA, these investigators demonstrated that plasma IαIp levels were significantly decreased in

severe septic patients compared to healthy controls. This decrease correlated with mortality suggesting that IαIp might have predictive value in septic patients. Lim, Y.P., et al., *Inter-trypsin inhibitor: decreased plasma levels in septic patients and its beneficial effects in an experimental sepsis model.* Shock, 2000. **13 (Suppl.)**: p. 161. *In-vivo* animal studies using a polymicrobial sepsis rat model of cecal ligation and puncture, showed that administration of IαIp produced dramatic improvements in survival rates compared to saline controls. *Yang S, et al., Administration of human inter-alpha-inhibitors maintains hemodynamic stability and improves survival during sepsis.* Crit Care Med. 2002 Mar;30(3):617-22. Taken together, the results strongly support the therapeutic potential of IαIp in the management of severe sepsis. Yet, there is no ready supply of IαIp for administration to septic patients. Thus, there is a clear need for fully functional, fully native IαIp that overcome the shortcomings of recombinant or blood-derived TPPs.

There are a number of patents and publications that describe immortalized cell lines: U.S. Patent No. 6,107,043 (Jauregui); U.S. Patent No. 5,665,589 (Harris); U.S. Patent App. No. 2002/0045262 A1 (Prachumsri); and International publication No. WO 99/55853 (Namba). However, to date, among other things, the prior art cell lines do not provide a means to safely, effectively, and cost efficiently perform the protein post-translational modifications, such as glycosylation, that are critical in the production of functional therapeutic plasma protein; produce simultaneously multiple therapeutic plasma proteins, especially factor VIII protein or factor IX; and maintain the continuous expression of active levels of cytochrome P450 enzyme in a serum-free media.

SUMMARY OF THE INVENTION

The present invention relates to a nontumorigenic, virally-immortalized human hepatocyte, that can be maintained in serum-free media, and produce endogenous plasma proteins, such as albumin, α-1 antitrypsin, blood clotting factors VIII and IX, and inter-α - inhibitor proteins (IαIp). In a preferred embodiment, the nontumorigenic, immortalized cell lines comprise the Fa2N-4 and Ea1C-35 cell lines deposited with the American Type Culture Collection (ATCC).

In a preferred embodiment of the present invention, the cell lines are derived from normal hepatocytes. Preferably, the cell lines are derived from normal human hepatocytes. More preferably, the cell lines are derived from cryopreserved normal human primary hepatocytes.

In another preferred embodiment of the present invention, the cell lines proliferate easily in media. Preferably, the cell lines proliferate easily in a serum-free media. More preferably, the cell lines proliferate easily in MFE (MultiCell Technologies Inc., Providence, RI, USA; XenoTech, LLC, Lenexa, KS, USA).

In another preferred embodiment of the present invention, the cell lines retain their hepatic functions in a serum-free media. Preferably, hepatic functions are the ability to continue to express enzymatic activity and produce proteins. More preferably, hepatic functions include the ability to continue to maintain cytochrome P450 enzymatic activities and produce fully-functional therapeutic plasma proteins in a serum-free media.

In another preferred embodiment of the present invention, the cell lines contain a substantially pure SV40 DNA. Preferably, the SV40 DNA encodes the wild type SV40 large T and small t antigens (TAg). More preferably, the DNA encodes the wild type TAg and does not encode other SV40 gene products.

In another preferred embodiment of the present invention, the cell lines continue to produce proteins. Preferably, the cell lines continue to naturally produce plasma proteins. More preferably, the cell lines continue to naturally produce therapeutic plasma proteins (TPP) comprising albumin, α-1-antitrypsin, factors VIII and IX, and inter-α-inhibitor proteins (IαIp).

In another preferred embodiment of the present invention, production of TPP by the cell lines is measured. Preferably, production of TPP by the cell lines is measured by detecting their presence in the serum-free media. More preferably, production of TPP by the cell lines is measured by detecting protein genes at the protein level rather than at the mRNA level.

In another preferred embodiment of the present invention, the cell lines produce TPP in serum-free media. Preferably, the cell lines simultaneously produce TPP out of the same fraction in serum-free media. More preferably, the cell lines simultaneously produce TPP out of the same fraction in serum-free media without the reoccurring risk of viral contamination.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a shows immunostaining of the Ea1C-35 immortalized hepatocyte cell line for large T antigen that confirms the integration of SV40DNA into genomic DNA of the immortalized cell.

Figure 1b shows immunostaining of cultured Fa2N-4 cells that demonstrates that the proliferating cells continue to express albumin.

Figure 1c shows the morphology of the immortalized cells shows well-defined nucleoli and granulated cytoplasm, which are characteristic features of normal primary hepatocytes.

Figure 2 shows an immunoblot showing induction of CYP3A4 consequent of treatment of Fa2N-4 and EA1C-35 with Rifampin (RIF), beta-naphtoflavone (BNF) and phenobarbital (PB). C is the untreated control. It should be noted that the upper band is nonspecific and that BNF, a CYP1A inducer does not induce CYP3A4 protein expression.

Figure 3 shows the following lanes: 1) Human Plasma; 2) Empty; 3) Culture Medium (Control); 4) Primary human hepatocytes (72 hr culture); 5) Ea1C-35 monolayer, 72 hrs culture; 6) Ea1C-35, roller bottle, 7-day culture; 7) Ea1C-35 roller bottle /14-day culture; 8) Fa2N-4 monolayer /72 hrs culture; 9) Fa2N-4 roller bottle /7-day culture; 10) Fa2N-4, roller bottle /14-day culture.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations and Terms

In accordance with the present invention and as used herein, the following terms and abbreviations are defined with the following meanings, unless explicitly stated otherwise. These explanations are intended to be exemplary only. They are not intended to limit the terms as they are described or referred to throughout the specification. Rather, these explanations are meant to include any additional aspects and/or examples of the terms as described and claimed herein.

The following abbreviations are used herein:

MCT = MultiCell Technologies

MFE = Multi-functional Enhancing media

TPP = therapeutic plasma proteins

IαIp = inter-alpha-inhibitor proteins

SV40 = simian virus 40 T antigen and t antigen

AAT = α-1-antitrypsin

The term "cell line" refers to a population or mixture of cells of common origin growing together after several passages *in vitro*. By growing together in the same medium and culture conditions, the cells of the cell line share the characteristics of generally similar growth rates, temperature, gas phase, nutritional and surface requirements. The presence of cells in the cell line expressing certain substances, for example albumin, can be ascertained, provided a sufficient proportion, if not all, of cells in the line are present to produce a measurable quantity of the substance. An

enriched cell line is one in which cells having a certain trait, e.g. expressing albumin, are present in greater proportion after one or more subculture steps, than the original cell line.

The term "clonal cells" are those, which are descended from a single cell. As a practical matter, it is difficult to obtain pure cloned cell cultures of mammalian cells. A high degree of cell purity can be obtained by successive rounds of cell enrichment. As used herein, a cell culture in which at least 80% of the cells possess a defined set of traits is termed a cloned cell culture. Preferably, a cell culture in which at least 90% of the cells possess a defined set of traits is termed a cloned cell culture. More preferably, a cell culture in which at least 98% of the cells possess a defined set of traits is termed a cloned cell culture.

The term "hepatocytes" refers to liver cells that are capable of considerable regeneration in response to loss of liver mass (e.g., through hepatotoxic processes, disease, or surgery), and constitute about 80% of the cell population of the liver. They are large polygonal cells measuring between 20-30 um. Hepatocytes have as many as 200-300 peroxisomes per cells, which are involved in the breakdown of hydrogen peroxide, produced in many of the general cytoplasmic metabolic activities. In addition, peroxisomes have specific oxidative functions in gluconeogenesis, metabolism of purines, alcohol and lipids. The smooth endoplasmic reticulum(sER) in hepatocytes contains enzymes involved in degradation and conjugation of toxins and drugs. Under conditions of hepatocyte challenge by drugs, toxins or metabolic stimulants, the sER may become the predominant organelle in the cells.

The term "immortalized" refers to the cell line that grows continually without senescence when cultured *in vitro* in a suitable growth medium.

The term "virally-immortalized" refers to hepatocytes being transfected with all or part of the viral genome of a wild type or mutant virus. Preferably, the virus is a DNA virus. More preferably, the virus is simian virus 40 (SV40).

The term "immortalization" is defined as the acquisition of an infinite life span. May be induced in finite cell lines by tranfection with telomerase, oncogenes, or the large T antigen of the SV40, or by infection with SV40. Immortilization is not necessarily a malignant transformation, though it may be a component of malignant transformation.

The term "metabolic activity" refers to the ability to process a potentially toxic compound, e.g., a drug or endogenous metabolite, into a less toxic or non-toxic compound.

The term "substantially pure" refers to a DNA which has been purified from the sequences which flank it in a naturally occurring state, i.e., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in the genome in which it naturally occurs, and which has been substantially purified from other components which naturally accompany the DNA, e.g., DNA which has been purified from the proteins which naturally accompany it in the cell.

The term "normal primary human hepatocyte" refers to a hepatocyte derived from a nondiseased human liver and maintained in vitro for a finite period when cultured in a suitable medium

The term "cryopreserved human hepatocyte" refers to a normal primary human hepatocyte that was cryopreserved prior to being cultured in a suitable medium.

Hepatocyte-derived proteins provide a safer, more reproducible approach for producing native plasma proteins for therapeutic applications. This finding is based upon MCT's data that demonstrates its proprietary, immortalized human hepatocyte cell lines, continue to produce inter-alpha-inhibitor proteins, a complex family of plasma proteins made by three different polypeptides that are produced from four different genes. Salier, J.-P., et al., *The inter-a-inhibitor family: from structure to regulation.* Biochem J, 1996. **351**: p. 1-9.

In contrast to heterologous proteins produced by genetic recombination in mammalian cells, such as Chinese Hamster Ovary cells, TPP derived from the cell lines of the present invention behave more normally since the secondary post-translational modifications required for complete function was preformed by the native hepatocyte manufacturing process. A significant advantage of using the cells of the present invention to produce TPP is that the producer cell line is of human origin and therefore leads to a more natural protein. Therefore, since a number of therapeutic plasma proteins (TPP) are synthesized by human hepatocytes, human hepatocyte-based expression systems of the cell lines of the present invention are used to produce TPP in their "native" form.

The inventors have developed a large number of proprietary immortalized human hepatocyte cell lines. The majority of these cell lines were created using

simian virus 40 (SV40) T antigen as the immortalization gene. This strategy was chosen because transfection of human cells with T antigen results in cell lifespan extension and frequently in nontumorigenic immortalization since the cells are semipermissive to viral infection. T antigen is a nuclear protein of 90,000 daltons.

Cascio, S., *Novel strategies for immortalization of human hepatocytes.* Artificial Orgs, 2001. 25: p. 529-538.

The multiple mechanisms of T antigen action are still under investigation, but many studies demonstrate that this viral protein binds to and inactivates Rb and p53, two key tumor suppressor genes of the host cell. Ludlow, J., *Interactions between SV40 large-tumor antigen and the growth suppressor proteins pRB and p53.* FASEB J, 1993. 7: p. 866-871.

While inactivation of Rb and p53 extends the lifespan of the cell, immortalization requires a secondary genetic event in order for the cell to escape senescence and proliferate indefinitely. The nature of this event is poorly understood, but occurs when the cells proceed through crisis. Most SV40 T antigen immortalized cell lines retain varying levels of the differentiated characteristics associated with the primary cell type and do not display tumorigenicity prior to extensive passage *in vitro*. Kuroki, T. and N. Huh, *Why are human cells resistant to malignant cell transformation in vitro?* Jpn J Cancer Res, 1993. 84: p. 1091-1100.

The normal human liver primary cells can be made to grow continuously by transfecting the cells with the T antigen gene of SV40 virus. Transfection or infection can be accomplished by use of a virus or a plasmid containing the T antigen gene of the SV40 virus.

Either transfection or infection may lead to transformation of the cell line. Other transformation vectors may also be useful, such as papilloma virus or Epstein Barr virus. The techniques for making continuous human cell lines are described in the following references: Grahm. F. L., Smiley J., Russell, W. C. and Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol., 36:59-72 (1977); Zur Hausen, H. Oncogenic herpes viruses In: J. Tooze (ed.), DNA tumor viruses, Rev. Ed. 2, pp 747-798. Cold Spring Harbor, N.Y., Cold Spring Harbor Press (1981); Popovic, M., Lange-Wantein, G., Sarin, P. S., Mann, D. and Gallo, R. C. Transformation of a human umbilical cord blood T-cells by human T-cell leukemia/lymphon virus (HTLV), Proc. Natl. Acad. Sci. USA; DiPaolo, J. A. Pirisi, I., Popeseu, N. C., Yasumoto, S., Poniger, J. Progressive changes induced in human and mouse cells by human Papillomavirus Type-16 DNA, Cancer Cells 5:253-257 (1987).

Post-translational modifications of therapeutic proteins may affect bioactivity, clearance rate *in vivo*, immunogenicity and/or stability. Plasma proteins secreted by our hepatocyte-based expression systems of the present invention behave more naturally than recombinant counterparts. For example, the inventors demonstrated that its immortalized human hepatocyte cell lines produce functional IαIp and therefore is a strong commercial source for this protein that cannot be produced by recombinant technology. Therefore, the inventors' production of IαIp in its "native" form leads to a more effective, safe, and cost effective solution to treating life threatening diseases such as sepsis.

As a result of the cell lines of the present invention, a sequential protein purification scheme generates multiple products similar to plasma-derived proteins without the

reoccurring risk of viral contamination. These hepatocyte-derived plasma proteins provide a safe, effective, and cost efficient strategy to commercially produce native plasma proteins, which overcomes the shortcomings of the prior art.

Creation of Immortalized Human Hepatocyte Cell Lines

Primary Cell Isolation

Digestion of donor, human liver was performed *in vitro* with pre-perfusion of oxygen-saturated, calcium-free buffer at 37°C. Pre-perfusion continued until the liver was blanched and followed by perfusion with oxygen-saturated, collagenase buffer until the liver was thoroughly digested (approximately 45 minutes).

To harvest cells, the liver was minced into 1 cm^2 pieces with the resulting suspension filtered through a #10 wire screen, then filtered again through a 253um nylon mesh. The suspension was centrifuged at 20xg for five minutes at 4°C to sediment intact parenchymal cells. The pellet was resuspended at 4°C and washed with washing buffer (3X) to remove all collegenase. The cell pellet was resuspended in 150ml tissue culture media to yield a final volume of 400-500ml with a density of $3\text{-}4x10^7$ cells/ml. Trypan blue and lactate dehydrogenase viability assessment was performed on aliquots of this suspension.

Cryopreservation of Primary Human Hepatocytes

The freshly isolated human hepatocytes isolated from donor liver as described above were washed with washing buffer three times by centrifuging at 50 x g for 5 minutes. The cell pellet was resuspended in chilled freezing medium (serum-free MFE medium: FBS:DMSO (8:1:1) at a final cell density of $5x\ 10^6$/mL. Aliquots of

the cell suspension were transferred to Nunc Cryovials (1.0 mL/1.5m cryovial, 4.5mL/5 ml cryovial). The cells in cryovials were equilibrated at 4°C for 15-30 minutes, then placed vials in a styrofoam container at -80°C for at least 3 hours. The vials were then plunged in LN_2.

Creation of Cell Lines

Cyropreserved human hepatocytes were rapidly thawed in a 42 degree Celsius water bath, washed and plated in MFE culture medium. Two days later cells the immortalizing gene was introduced by lipofection-mediated transfection. The Ea1C-35 cell line was derived from transfection with an immortalization vector containing the 2.5kb early region of the SV40 genome, which encompasses both the large-T and small-t antigens, and whose expression is driven by the SV40 early promoter. This early region was inserted into the Stratagene pBluescript SK vector backbone and was named pBlueTag. Neomycin resistance was conferred on the transfected cells as a selectable marker by co-transfection of a neo plasmid. Clones were initially selected based on their ability to grow in G418 containing media. The Ea1C-35 cell line was established and maintained in serum-free containing media, CSM.

The Fa2N-4 cell line was immortalized via lipofection-mediated transfection with a single immortalization vector. The early region of the SV40 genome, contained in the pBlueTag vector, was inserted into a backbone based upon the InvivoGen pGT60mcs plasmid and was named pTag-1. The T-antigen coding region is under the influence of a hybrid hEF1-HTLV promoter. The vector also encodes a hygromycin resistance gene as a drug selectable marker. Clones were selected based on their ability to grow in hygro containing media. The Fa2N-4 cell line was established and maintained in MFE.

The Fa2N-4 cell line was deposited under the terms of the Budapest Treaty at the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md., on October 6, 2003. The Ea1C-35 cell line was deposited under the terms of the Budapest Treaty at the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md., on October 6, 2003.

UTILITY

Examples of Uses

Utility of Cell Lines

(1) Identification of potential chemotherapeutic drugs: These cells are useful for screening chemicals suitable for the treatment of cancer and related diseases, by growing them *in vitro* in medium containing the chemical to be tested and then, after a suitable period of exposure, determining whether and to what extent cytotoxicity has occurred, e.g. by trypan blue exclusion assay or related assays (Paterson, Methods Enzymol, 58:141 (1979)), or by growth assays such as colony forming efficiency (MacDonald et al, Exp. Cell. Res., 50:417 (1968)), all of which are standard techniques well known in the art.

(2) Investigation of the controls of differentiation and identification of chemical and biological agents that induce terminal differentiation. Chemical and biological substances are screened for their ability to induce terminal differentiation by adding them to the growth medium of these liver cells and then after a suitable period of time, determine whether a complex of changes, including cessation of DNA synthesis, and production of liver specific proteins (as determined by in situ hybridization

techniques) occurs. Induction of terminal differentiation may be an effective way of controlling the growth of cancer.

(3) Studies of metabolism of carcinogens and other xenobiotics: Carcinogens and other xenobiotics may be added to the growth medium of these cells and the appearance of metabolic products of these compounds may be monitored by techniques such as thin layer chromatography or high performance liquid chromatography and the like, and the interaction of the compounds and/or their metabolites with DNA is determined.

(4) Studies of DNA mutagenesis: Substances known or suspected to be mutagens may be added to the growth medium of the cells and then mutations may be assayed, e.g., by detection of the appearance of drug resistant mutant cell colonies (Thompson, Methods Enzymol, 58:308, 1979).

(5) Studies of chromosome damaging agents: Substances known or suspected to cause DNA or chromosomal damage may be added to the culture medium of these cell lines, and then the extent of chromosomal damage may be measured by techniques such as measurement of the frequency of sister chromatic exchange (Latt et al. In: Tice, R. R. and Hollaender, A., Sister Chromatic Exchanges, New York: Plenum Press, pp, 11 ff. (1984)).

Protocols for Detection of BrdUrd Substitution into Metaphase Chromosomes

1. 33258 Hoechst Fluorescence (S. A. Latt et al., Proc. Natl. Acad. Sci. USA 70:3395 (1973); S. A. Latt et al., Cytochem. 25:913 (1977))

a. Staining. Slides are successively dipped in PBS (0.14 M NaCl, 0.004 M KCl, 0.01 M phosphate, PH 7.0) (5'), 0.5 .mu.g/ml 33258 Hoechst in PBS (10'), PBS (1'), PBS (5'), and H.sub.2 O (two to three changes). Stock solutions of dye (50 .mu.g/ml) in H.sub.2 O can be stored at 4.degree. C. in the dark for at least 2 weeks. Samples of 33258 Hoechst were originally obtained from Dr. H. Loewe, Hoechst AG, Frankfurt, Germany, although the dye can now be purchased from Calbiochem.

b. Observation. Microscopic observation of 33258 Hoechst fluorescence is guided by the position of the high wavelength absorption band of the dye-DNA complex (maximal near 350 nm and appreciable up to or slightly beyond 425 nm). While excitation under dark-field conditions is certainly possible, excitation with incident illumination, using a UG-1 (360 nm peak) bandpass and TK 400 (reflect .ltoreq.400 nm) dichroic mirror is especially convenient and effective. A 460 nm high wavelength pass filter in the observation pathway removes most unwanted exciting light from the fluorescence, which peaks near 475 nm.

For optimal quenching of dye fluorescence by incorporated BrdUrd, the slides are mounted in a buffer of moderate ionic strength at or slightly above neutrality, for example, pH 7.5 McIlvaine's buffer. Primarily because of the specificity of the stain, under these conditions, for (A-T rich) DNA, and because the free dye has very weak fluorescence, slides observed as described above show little background fluorescence. However, the fluorescence of 33258 Hoechst bound to BrdUrd-substituted chromatin fades rapidly, and photography requires speed. Using a microscope with incident illumination, acceptable photographs can be obtained in 5-10 sec (e.g., Kodak Tri-X

film). Reduction of the mounting medium pH shifts the dye fluorescence color from blue toward green or yellow, and the fluorescence fades less rapidly; but specific quenching due to BrdUrd substitution is decreased.

2. 33258 Hoechst plus Giemsa (adapted from P. Perry and S. Wolff, Nature 261:156 (1974); S. Wolff (1981), Measurement of sister chromatid exchange in mammalian cells. In DNA Repair: A Laboratory Manual of Research Procedures, Volo 1, Part B (E. C. Friedberg and P. C. Hanawalt, Eds.), Dekker, N.Y.)

a. Staining. Slides are stained with 10.sup.-4 M 33258 Hoechst in one-third strength PBS (or a comparable buffer with a pH near 7). A cover slip is applied and the slides are then placed in a Petri dish containing excess buffer to ensure that the slides remain moist, and the slides are exposed to light for a period of time that depends on the illuminating conditions. For example, approximately 6-12 hours of exposure is sufficient after the slides are positioned 6 cm from a Sylvania 20 watt cool white bulb (Latt et al., Cytochem. 25:913 (1977)). The slides are then rinsed in H.sub.2 O, incubated at 65.degree. C. in 2 x SSC buffer (0.30 M NaCl, 0.03 M Na citrate, pH7) for 15 min., rinsed thoroughly with H.sub.2 0, and stained with Giemsa as before. Contrast can be heightened by increasing the time during which slides are exposed to light.

b. Observation. For photographs slides can be mounted in a standard embedding mediums or immersion oil can be applied directly to the slide without a cover slip. A 544 nm interference filter can be used to enhance contrast, and the optical image is recorded using, for example, High Contrast Copy film.

While there is a wealth of methods for differentiating between sister chromatids, a few simple techniques can suffice for most studies. Representative techniques, employing 33258 Hoechst fluorescence or 33258 Hoechst followed sequentially by illumination, SSC incubation, and Giemsa staining are detailed. Procedural details for SCE analysis have also been presented by (Wolff, S. (1981), Measurement of sister chromatid exchange in mammalian cells. In DNA Repair: A Laboratory Manual of Research Procedures, Vol. 1, Part B (E. C. Friedberg and P. C. Hanawalt, Eds.), Dekker, N.Y.) It should be noted that other techniques, related to those described above, can work equally well and DNA damage can be determined by the measurement of unscheduled DNA synthesis (Mirsalis, J. C. Banbury report vol. 13, p 83-99 (1982). The procedures for this assay have been previously described (J. C. Mirsalis et al. Environ. Mutagen 4 (in press)). Fischer-344 rats are treated with chemicals by a suitable route of exposure, and at selected times after treatment, their livers are perfused with a collagenase solution. A single-cell suspension of hepatocytes is obtained by combing out the cells of the perfused liver into a petri dish containing collagenase solution, Cells are seeded into culture dishes containing coverslips and Williams Medium E supplemented with 10% fetal bovine serum, allowed to attach to the coverslips, and incubated with a solution of 10 .mu.Ci/ml .sup.3 H-thymidine (.sup.3 H-dT) for 4 hours. Following overnight incubation (14-18 hr) in 0.25 mM unlabeled thymidine, cells are swelled, fixed, and washed; coverslips are mounted on microscope slides and dipped in Kodak NTB-2 photographic

emulsion. After being exposed for 12-14 days, slides are developed and the cells are stained.

Quantitative autoradiographic grain counting is accomplished using a colony counter interfaced to a microscope via a TV camera; data are fed directly into a computer. Fifty morphologically unaltered cells from a randomly selected area of the slide are counted. The highest count from three nuclear-sized areas over the cytoplasm and adjacent to the nucleus is subtracted from the nuclear count to give the net grains/nucleus (NG). The percentage of cells in repair indicates the extent of damage throughout the liver and is calculated as those cells exhibiting>5 NG.

(6) Studies of malignant transformation by chemical, physical and viral agents, and transferred genes including oncogenes and high molecular weight genomic DNA from tumors, using standard assays such as anchorage independent growth or tumor formation in athymic nude mice. For example, a cloned viral oncogene N-ras (an oncogene present in many liver cell cancers) can be introduced into the hepatocyte cells using strontium phosphate transfection. The subsequent ability of the newly transfected cells to form tumors in mice as well as grow in an anchorage-independent fashion can be assessed.

(7) Use of cells altered by transfer of oncogenes as in paragraph (6) above to screen for potential chemotherapeutic agents (by the techniques described in paragraph (1) above) especially those which may be specific for cells transformed by the activation of particular oncogenes or combination of oncogenes.

(8) Studies of cellular biochemistry, including changes in intracellular pH and calcium levels, as correlated with cell growth and action of exogenous agents including but not limited to those described in paragraphs (1) through (7) above. To study intracellular pH and calcium levels, cells in suitable culture vessels are exposed to fluorescent indicator dyes and then fluorescence emissions are detected with a fluorescence spectrophotometer (Grynkiewicz et al, J. Biol. Chem., 260:3440-3450 (1985)).

(9) Studies of cellular responses to growth factors and production of growth factors: Identification and purification of growth factors important for growth and differentiation of human liver hepatocyte cells. These cells are particularly useful for such an application since they grow in serum-free media. Therefore, responses to growth factors can be studied in precisely defined growth medium and any factors produced by the cells may be identified and purified without the complication of the presence of serum.

(10) Use of recombinant DNA expression vectors to produce proteins of interest. For example, the gene encoding a protein of therapeutic value may be recombined with controlling DNA segments (i.e. containing a promoter with or without an enhancer sequence), transferred into the cell (e.g., by strontium phosphate transfection) and then the protein produced may be harvested from the culture supernatant or a cellular extract by routine procedures well known in the art.

(11) Studies of intracellular communication e.g., by dye scrape loading assays, to determine whether the cells growing *in vitro* have the ability to communicate via gap

junctions. The cultures may be scraped, e.g., with a scalpel, in the presence of a fluorescent dye in the growth medium. Cells at the edge of the wound are mechanically disrupted and therefore take up dye; whether intercellular communication has occurred may be ascertained by determining whether cells distant from the wound also contain dye.

(12) Characterization of cell surface antigens: The cells are incubated with an antibody against the cell surface antigen of interest, and then reacted with a second antibody, which is conjugated to a fluorescent dye. The cells are then evaluated using a fluorescence activated cell sorter to determine whether they are fluorescent and therefore posses the cell surface antigen.

(13) Cell-cell hybrid studies for identification of tumor suppressor activity (Stranbridge et al, Science, 215:252-259 (1982)). To determine whether these cell lines contain tumor suppressor genes, they are fused to malignant tumor cells. The presence of tumor suppressor genes is indicated by loss of malignancy e.g., as detected by loss of ability to form tumors in athymic nude mice, in the hybrid cells.

(14) Identification of novel genes, including transforming genes in the naturally occurring cancer described in paragraph (6) above, growth factor genes as described in paragraph (9) above, tumor suppressor genes as described in paragraph (13) above, using standard molecular biological techniques (Davis et al, Methods in Molecular Biology, New York: Elsevier (1986)) and techniques such as cDNA subtraction cloning and similar processes.

(15) Growth of replicating hepatitis virus (as e.g., HBV, non-A non-B, HAV and other livertropic virus, e.g., CMV). Establishment of a clonal cell line of human liver hepatocyte cells containing replicating Hepatitis virus using methods of transfection established for human liver cancer cells lines (Sells, M. A. et al, Proc. Natl. Acad. Sci., 84:444-448). Using human liver hepatocyte lines, which contain HBV, the ability of HBV alone as well as in conjunction with chemical liver carcinogens such as aflatoxin B, can be evaluated for malignant transformation using anchorage independent growth assays as well as growth in athymic nude mice. Cell--cell hybrid techniques similar to those in paragraph (13) can be used to evaluate possible inactivation of tumor suppressor genes by fusion with malignant cells before and after HBV transfection.

The screening kits are easily assembled as other screening kits containing cell lines with other conventional components and labeling instructions for performing the test.

(16) The immortalized cells may be used as a way of expanding cells for liver transplant and liver function assist devices, both implanted and extracorporeal. Also, these cells can have additional genes transfected/infected into them for organ transplant for therapy of inherited metabolic disorders, especially those diseases associated with hepatic degradation (i.e., certain diseases are due to a deletion or abnormality of a particular gene). This gene could then be transfected into our cells, and the cells then expanded for organ transplant.

(17) Studies of cytotoxicity of drugs, carcinogens, xenobiotics: Drugs, carcinogens, xenobiotics may be added to the growth medium of the cells and the viability of the

cells as a function of time of exposure may be ascertained using gene expression profiling, dye exclusion, enzyme leakage, colony forming efficiency, etc. assays.

(18) Studies of gene expression: drugs, chemicals, new chemical entities, etc may be added to the culture medium of the cells and changes in gene expression as a function of exposure may be monitored using RNA and protein expression as biological endpoints. Changes may reflect either induction or inhibition of specific genes. For example, cells may be cultured with drugs, chemicals, new chemical entities, etc to identify those agents that modulate the expression of drug metabolism enzymes including but not limited to cytochrome P450s designated CYP3A4 or CYP1A2, the multi drug resistance gene, biliary transporters, glucuronyl transferases, glutathione transferases, sulfatases, etc.

(19) Studies of liver parasites: The cultured cells could prove efficacious for studying the life cycle of parasites that invade hepatocytes.

(20) Production of hepatocyte –derived proteins. Cells maintained in suitable medium will naturally express proteins such has blooding clotting factors (e.g. factor VIII and Factor IX), α-1-antitrypsin, growth factors, etc that may be purified and used.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Methods

Examples

The following examples are provided by way of describing specific embodiments of the present invention without intending to limit the scope of the invention in any way.

Example 1

Characterization of Immortalized Human Hepatocytes

Over 100 human hepatocyte clonal cell lines were established by transfecting human hepatocytes with the simian virus 40 large T and small antigen genes under control of the SV40 early promoter. Two cell lines designated Ea1C-35 and Fa2N-4 are described.

Both cell lines were created by lipofection-mediated transfection of primary cryopreserved human hepatocytes with vectors containing the SV40 largeT and small t antigens. The Ea1C-35 cell line was derived from transfection of cryopreserved human hepatocytes with an immortalization vector containing Blue Tag, a recombinant plasmid containing the early region of wild-type SV40. The Blue Tag vector was constructed as follows: PBR/SV (ATCC) was digested with restriction enzymes KpnI and BamHI to release a 2338 bp fragment (277-2516 bp) containing the SV40 early promoter and the coding regions from small t and large T antigens. This KpnI/BamHI fragment was inserted into the Bluescript SK vector (Strategene) to produce Blue Tag; a Bluescript based vector that uses the SV40 promoter to drive T antigen expression. This early region was inserted into the Stratagene pBluescript SK vector backbone and was named pBlueTag. Neomycin resistance

was conferred on the transfected cells as a selectable marker by co-transfection of a neo plasmid. Clones were initially selected based on their ability to grow in G418 containing media. The Ea1C-35 cell line was established and maintained in MCT's proprietary serum containing media, CSM.

The Fa2N-4 cell line was immortalized via lipofection-mediated transfection with a single immortalization vector. The early region of the SV40 genome, contained in the pBlueTag vector, was inserted into a backbone based upon the InvivoGen pGT60mcs plasmid and was named pTag-1. The T-antigen coding region is under the influence of a hybrid hEF1-HTLV promoter. The vector also encodes a hygromycin resistance gene as a drug selectable marker. Clones were selected based on their ability to grow in hygro containing media. The Fa2N-4 cell line was established and maintained in MFE.

Example 2

Expression of Liver Specific Transcription Factors

Since retention of liver specific transcription factors is a prerequisite for expression of hepatic functions, clonal cell lines were initially screened by RT-PCR using primers for human HNF1, HNF3, HNF4α, HNF4γ and C/EBP and albumin. Briefly, total RNA was prepared from 10^6 cells of each clonal cell line using the micro-isolation method of Brenner et al. (55). 50 μg of E. coli rRNA (Sigma) was used as a carrier to facilitate the isolation of RNA from a small number of cells. RT-PCR reactions were carried out using the Perkin Elmer Cetus, GeneAmp RNA PCR

Kit. One μg of total RNA was reverse transcribed using random hexamers and M-MLV reverse transcriptase according to the supplier's instructions. The PCR reaction was carried out using oligonucleotide primers that defined nucleotide fragments unique for each transcription factor. The primers were commercially synthesized and purified by Cruachem (Fisher Scientific). The PCR reaction was carried out for 30 cycles using an annealing temperature of 58°C for 1 min. The PCR products were visualized in a 1% agarose gel after staining with ethidium bromide. Positive control samples included RT-PCR analysis of total RNA of freshly isolated human hepatocytes (not shown). Both cell lines expressed all five hepatocyte associated transcription factors, as shown below in Table 1. Albumin production was measured as an indicator of hepatocyte specific gene expression. As shown below in Table 1, both cell lines secrete albumin into the serum free conditioned medium as detected by ELISA assay using an antibody that recognizes human albumin.

Table 1

Clones	HNF - 1	HNF – 3α	HNF – 4α	HNF – 4γ	hC/EBP	Albumin (ug/mg protein)[1]
Fa2N-4	+	+	+	+	+	2.79
Ea1C-35	+	+	+	+	+	0.3

Example 3

SV40 Mediated Proliferative Activity

Primary human hepatocytes have limited proliferative activity when cultured. In order to overcome this characteristic, SV40 large T and small antigens were introduced into the genome. The resulting clonal cell lines, Fa2N-4 and Ea1C-35 have subsequently been maintained in culture for up to 18 months. Both immortalized lines grow and function when maintained in MFE medium and can be cryopreserved and banked. Indirect immunofluorescent staining using a polyvalent antibodies against large T antigen and albumin demonstrated that the cell lines continue to express the nuclear localized immortalizing gene (Figure 1a) as well as express a hepatocyte specific gene characteristic of differentiated function (Figure 1b). The morphology of the Ea1C-35 cell line is shown below (Figure 1c).

Example 4

Drug Metabolism Data

Both cell lines continue to catalyze Phase I (cytochrome P450) and Phase II conjugative reactions in monolayer cultures based on the metabolism of model substrates. One of the most important Phase I enzymes is CYP3A4, which is responsible for the metabolism of approximately 50% of all drugs. The expression of CYP3A4 can be modulated by many factors including multiple drug intake that may induce or inhibit the overall expression of this P450. Therefore the effective therapeutic dose of a drug is determined in part by CYP3A4 expression.

CYP3A4 modulators can be identified by monitoring the transcriptional responsiveness of the gene and by measuring enzymatic activity towards model substrates (i.e. testosterone). For example, transcriptional responsiveness to prototypical pharmacological CYP3A4 inducers (i.e. rifampin) can be assayed by the reverse transcription polymerase chain reaction (RT-PCR) using specific primers to detect CYP3A4 mRNA. Rifampin-induced CYP3A4 enzymatic activity can also be measured by the production of the 6β-OH-testosterone metabolite when cells are incubated with testosterone. As shown below in Table 2, the Fa2N-4 cell line is more sensitive to CYP inducers than the Ea1C-35 cell line.

In order to demonstrate that the cell lines continue to express Phase II conjugating enzymes, cells were exposed to acetaminophen for 24 hours and conditioned culture medium was collected and analyzed for the production of acetaminophen glucuronide or sulfate conjugates. These results indicate that both pathways are intact.

Table 2 Characteristics of the Fa2N-4 and Ea1C-35 cell lines.

Cell line	Rifampin treated CYP3A4 (mRNA fold induction)[1]	Control (ug 6β-OH testosterone/ mg protein)[2]	Rifampin (ug 6β-OH testosterone/ mg protein)[2]	Acetamenophe glucuronide (ug/mg protein)	Acetamenophen sulfate (ug/mg protein)
Fa2N-4 (p13)	15.4	5.44	15.28	20.9	16.1
Ea1C-35 (p29)	2.2	4.53	9.25	15	21.5

[1]Cells were exposed to vehicle or rifampin for 72 hours. Data is expressed relative to vehicle treated controls.

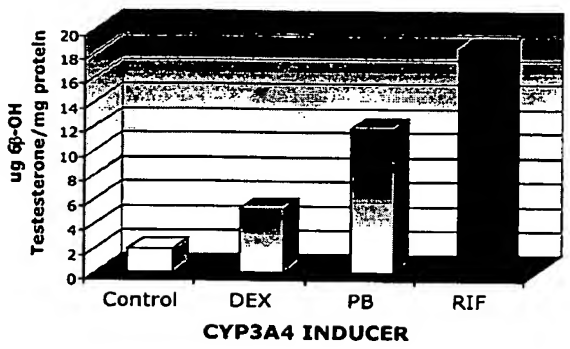
[2]Cells were exposed to vehicle or rifampin for 72 hours and then incubated with testosterone for 24 hours. Production of the 6β-OH-testosterone metabolite was quantitated by HPLC analysis and data is expressed per mg total cell protein.

Example 5

Use of Immortalized Hepatocytes to Identify and Rank CYP Inducers

Two lines of evidence indicate that immortalized human hepatocytes can be employed to identify and rank CYP3A4 inducers based on 'induction potency'. First, exposing Fa2N-4 cells to rifampin (10 μM) results in a greater production of the 6-β testosterone metabolite than treating cells with weaker CYP3A4 inducers such as dexamethazone (50 uM) or phenobarbital (1mM), as shown below in Table 3. Secondly, immunoblot analysis demonstrated that exposure of each of the cell lines to rifampin or phenobarbital for 48-72 hours increased expression of CYP3A4 protein in comparison to vehicle-treated controls; however, exposure to rifampin resulted in a greater increase expression of CYP3A4 protein.

Table 3. Inducibility of testosterone metabolism after treating Fa2N-4 cells with different CYP3A4 inducers.



Example 6

Expression of Plasma Proteins By Fa2N-4 and Ea1C-35 Cell Lines

The well-differentiated nature of these cell lines is further supported by their continued secretion of adult hepatocyte function specific plasma proteins (Figure 3). Culture medium was harvested from Fa2N-4 and Ea1C-35 cells seeded into either 60 mm plates or roller bottles and analyzed by Western blot analysis. Medium was concentrated 50X by ultrafiltration and 40 μg of total protein was loaded per lane except for albumin (10 μg total protein/lane). Blots were incubated with either monoclonal or affinity purified polyclonal antibodies against albumin, α–1-antitrypsin, Factor VIII and Factor IX and visualized using secondary antibodies

conjugated to horseradish peroxidase followed by incubation with DAB substrate. As shown below in Figure 3, both cell lines continue to express albumin, α-1-antitrypsin, and Factor IX. The expression of Factor VIII was variable and highly dependent on cell line and culture conditions. There was heterogeneity in the processing of Factor IX, an observation also seen in the human plasma-derived protein.

RESULTS

Taken together, all the above examples strongly indicate that the two immortalized human hepatocyte cell lines maintain many functional attributes characteristic of hepatocytes *in vivo* and is an invaluable *in vitro* system to produce therapeutic plasma proteins.

The inventions illustratively described herein can suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the future shown and described or any portion thereof, and it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions herein disclosed can be resorted by those skilled in the art, and that such modifications and variations are considered to be

within the scope of the inventions disclosed herein. The inventions have been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the scope of the generic disclosure also form part of these inventions. This includes the generic description of each invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised materials specifically resided therein.

In addition, where features or aspects of an invention are described in terms of the Markush group, those schooled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. It is also to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of in the art upon reviewing the above description. The scope of the invention should therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent publications, are incorporated herein by reference.

We claim:

1. A virally-immortalized human hepatocyte, said hepatocyte

(a) being derived from a normal human liver cell;

(b) is nontumorigenic;

(c) having the ability to be maintained in serum free media; and

(d) naturally produces endogenous plasma proteins, wherein said plasma proteins consists of albumin, α-1 antitrypsin, blood clotting factors VIII and IX, and inter-α -inhibitor proteins (IαIp).

2. The hepatocyte according to claim 1, wherein said hepatocyte is Fa2N-4

3. The hepatocyte according to claim 1, wherein said hepatocyte is derived from primary cryopreserved human hepatocytes.

4. The hepatocyte according to claim 1, wherein said hepatocyte is immortalized by a substantially pure simian virus 40 (SV40) DNA.

5. The hepatocyte according to claim 3, wherein said DNA encodes wild type SV40 large T and small t antigens (TAg).

6. The hepatocyte according to claim 3, wherein said hepatocyte comprises substantially pure tumor suppressor-encoding DNA.

7. The hepatocyte according to claim 3, wherein said pure tumor suppressor-encoding DNA comprises a substantially pure human Rb-encoding DNA.

8. The hepatocyte according to claim 3, wherein said hepatocyte comprises a substantially pure human p53 encoding DNA.

9. The hepatocyte according to claim 1, wherein said serum free media is MCT's proprietary serum free media.

10. The hepatocyte according to claim 1, wherein said plasma proteins consists of at least a significant amount of albumin.

11. The hepatocyte according to claim 1, wherein said plasma proteins consists of at least a significant amount of α-1 antitrypsin.

12. The hepatocyte according to claim 1, wherein said plasma proteins consists of at least a significant amount of blood clotting factors VIII and IX.

13. The hepatocyte according to claim 1, wherein said plasma proteins consists of at least a significant amount of inter-α -inhibitor proteins (IαIp).

14. A virally-immortalized human primary hepatocyte cell line Fa2N-4.

15. A virally-immortalized human hepatocyte, said hepatocyte

(a) being derived from a normal human liver cell;

(b) is nontumorigenic;

(c) having the ability to be maintained in serum free media;

(d) having the ability to form the acetaminophen conjugate; and

(e) naturally produces endogenous plasma proteins, wherein said plasma proteins consists of albumin, α-1 antitrypsin, blood clotting factors VIII and IX, and inter-α -inhibitor proteins (IαIp).

16. The hepatocyte according to claim 14, wherein said hepatocyte is Ea1C-35.

17. The hepatocyte according to claim 14, wherein said hepatocyte is derived from primary cryopreserved human hepatocytes.

18. The hepatocyte according to claim 14, wherein said hepatocyte is immortalized by a substantially pure simian virus 40 (SV40) DNA.

19. The hepatocyte according to claim 16, wherein said DNA encodes wild type SV40 large T and small t antigens (TAg).

20. The hepatocyte according to claim 16, wherein said hepatocyte comprises substantially pure tumor suppressor-encoding DNA.

21. The hepatocyte according to claim 16, wherein said pure tumor suppressor-encoding DNA comprises a substantially pure human Rb-encoding DNA.

22. The hepatocyte according to claim 16, wherein said hepatocyte comprises a substantially pure human p53 encoding DNA.

23. The hepatocyte according to claim 14, wherein said serum free media is MCT's proprietary serum free media.

24. The hepatocyte according to claim 14, wherein said plasma proteins consists of at least a significant amount of albumin.

25. The hepatocyte according to claim 14, wherein said plasma proteins consists of at least a significant amount of α-1 antitrypsin.

26. The hepatocyte according to claim 14, wherein said plasma proteins consists of at least a significant amount of blood clotting factors VIII and IX.

27. The hepatocyte according to claim 14, wherein said plasma proteins consists of at least a significant amount of inter-α -inhibitor proteins (IαIp).

28. A virally-immortalized human primary hepatocyte cell line Ea1C-35.

ABSTRACT OF THE DISCLOSURE

This invention relates to virally-immortalized hepatocyte cell lines, which are derived from a normal primary human liver cell, have the ability to proliferate in a serum-free media, are nontumorigenic, and produce proteins.

Figure 1a

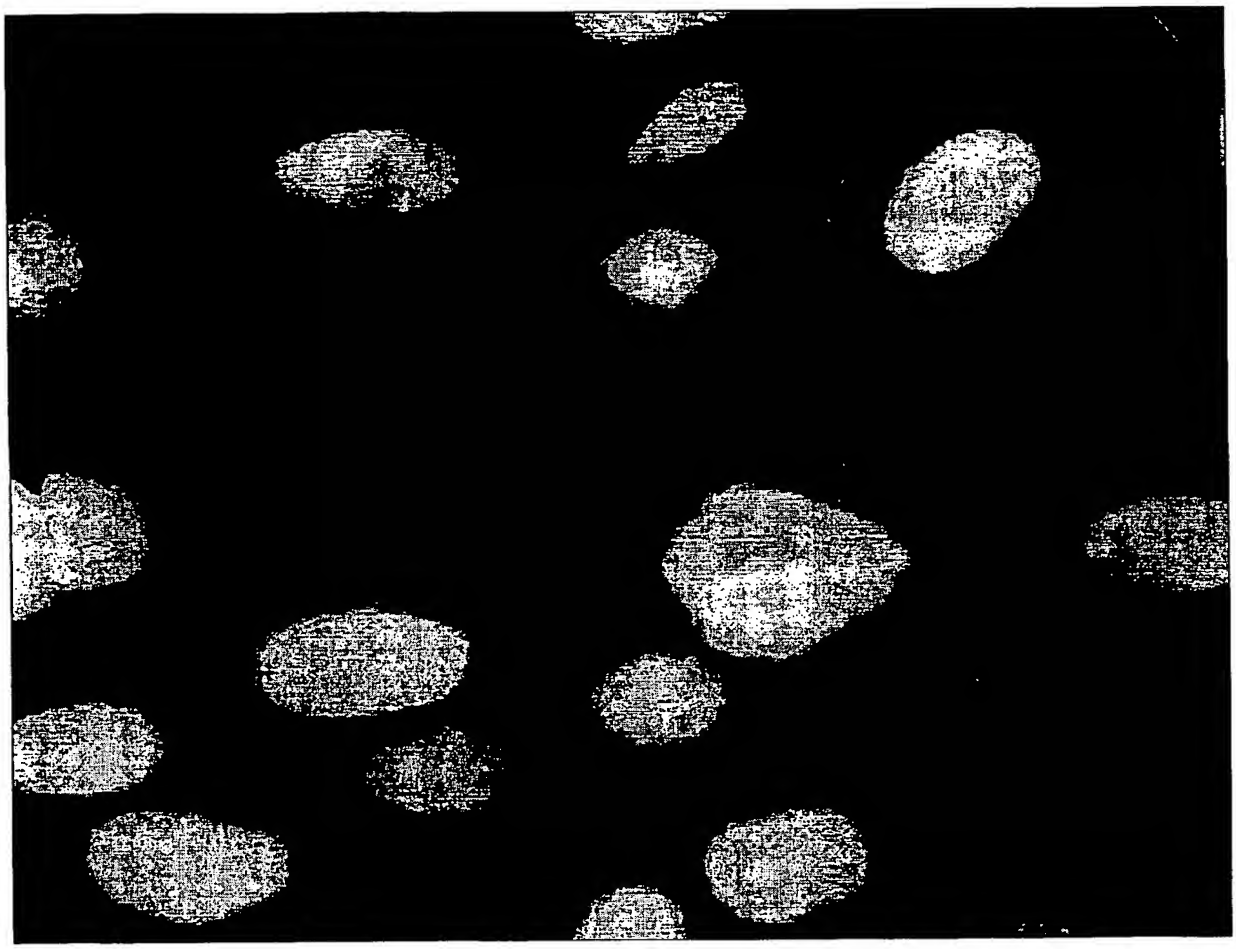


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Figure 1b

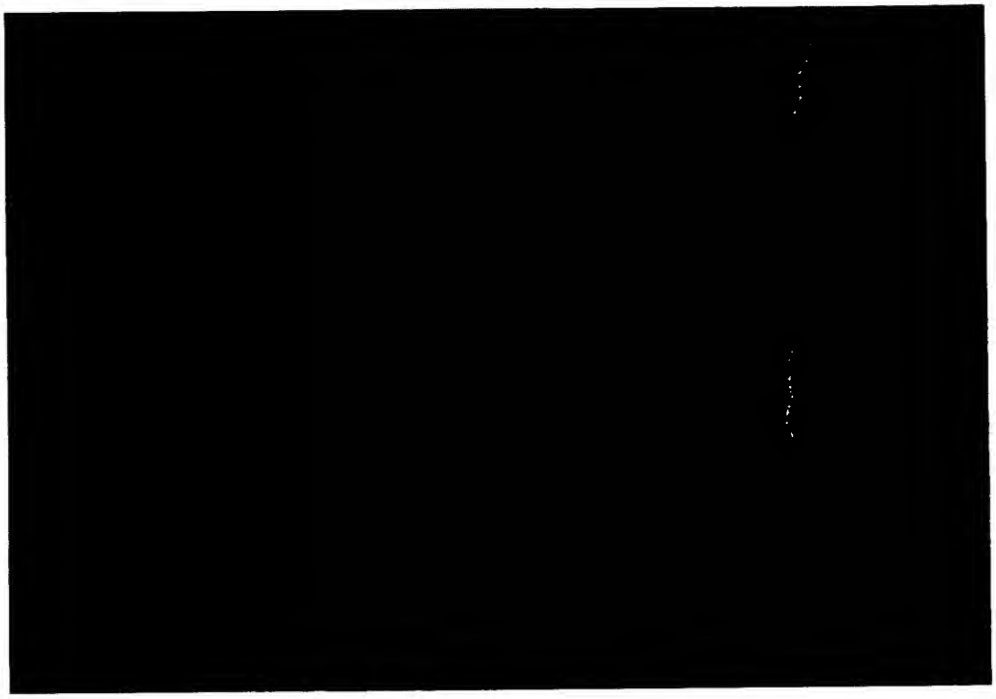

Figure 1c

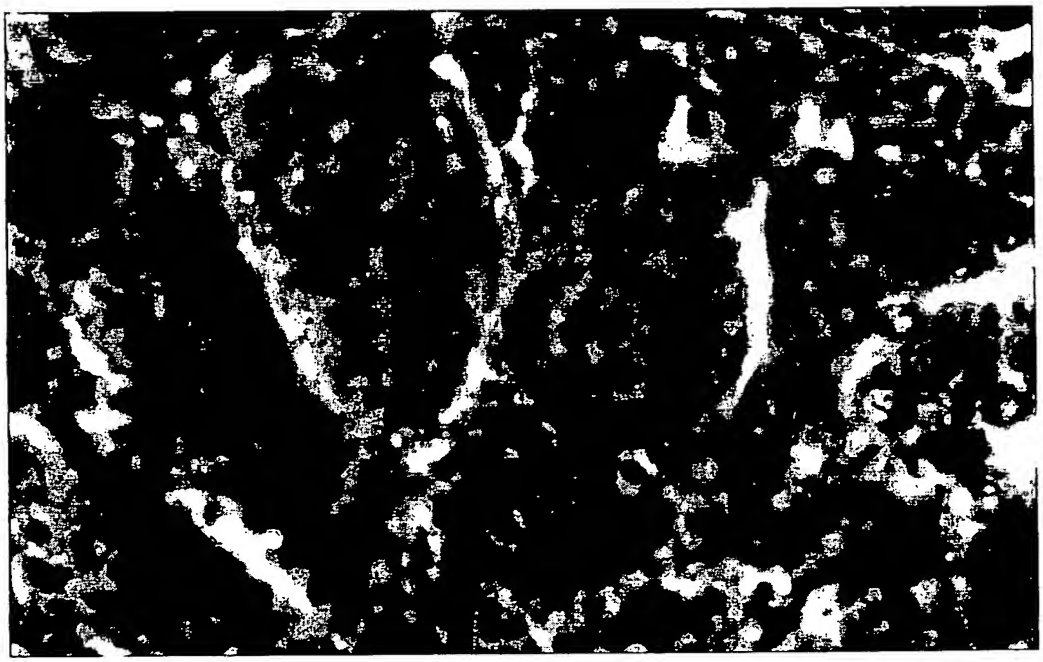

Figure 2

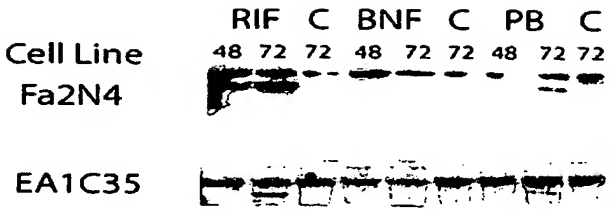

Figure 3